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VIABILITY AND COLONY MORPHOLOGY VARIATION OF *RHODOCOCCUS RHODOCHROUS* CNMN-AC-05 IN THE PRESENCE OF MAGNETITE NANOPARTICLES

O. Postolachi[1], I. Rastimesina[1], V. Josan[1], T. Gutul[2]

[1]Institute of Microbiology and Biotechnology,
1 Academiei Str., Chişinău, MD-2028, Republic of Moldova
[2]Institute of Electronic Engineering and Nanotechnologies 'D.Ghitu',
3/3 Academiei Str., Chişinău, MD-2028, Republic of Moldova
e-mail: oleseap@yahoo.com

In recent decades the use of nanotechnologies in the remediation of xenobiotic substances has proven its effectiveness, but not its safety. Nanoparticles often accumulate in the remedied environment, having, over time, toxic effects on living organisms. In this context, research on the vital activity of microorganisms and their interaction with nanoparticles is of major importance. ***Aim*** *of the research was to determine the influence of Fe_3O_4 nanoparticles, obtained by different ways (laboratory method and synthesis in the reactor) on the viability and colony morphology of Rhodococcus rhodochrous CNMN-Ac-05 strain.* ***Methods****. Encapsulated magnetite (Fe_3O_4) nanoparticles were synthesized by chemical co-precipitation method, using iron(II) sulfate and iron(III) chloride in the presence of poly-N-vinylpyrrolidone, used as a stabilizer. Fe_3O_4SR (Synthesis in the Reactor) was produced in the multifunctional reactor VGR-50, at the same conditions. Cell biomass was determined on the spectrophotometer by the optical density at 540 nm, with subsequent recalculation to cell dry weight according to the calibration curve. The cell dry weight was determined by gravimetric method. The morphological features of the rhodococci colonies were described according to the standard microbiological method.* ***Results****. It was established that magnetite nanoparticles in concentrations of 1–100 mg/L were not toxic to the R. rhodochrous strain, had a positive effect on the viability of rhodococci by stimulating the growth of biomass, regardless of their concentration and the method of their synthesis. In the presence of Fe_3O_4 nanoparticles the population dissociated to S1, S2, R1, R2 forms, and S-R type of colonies, while the basic morphological features of R. rhodochrous colonies corresponded to type S1.* ***Conclusions****. The optimal concentration of magnetite nanoparticles, which stimulated the growth and development of R. rhodochrous was 25 mg/L for Fe_3O_4 and 50 mg/L Fe_3O_4SR. At all concentration of Fe_3O_4 nanoparticles the main colony morphotype of the rhodococci was smooth S1-type; the new types of colonies represented only 0.1–0.6 % of the population, and the lowest degree of variability corresponded with the highest colony-forming units index.*

Keywords: rhodococci, magnetite NPs, colony morphology.

Actinobacteria of the genus *Rhodococcus* are widespread in the natural environment, including soils, rocks, boreholes, groundwater, marine sediments, animal dung, insect guts, even in healthy and diseased animals and plants [1]. *Rhodococcus* spp. is able to survive in the presence of high doses of toxic compounds, as well as under desiccation conditions, carbon starvation, a wide range of temperatures (from 4 to 45 ^{0}C), UV irradiation and osmotic stress [2–5]. *Rhodococcus* strains are often isolated from environments where hydrocarbons are present [6].

The ability of these non-sporulating bacteria to survive in hostile environmental conditions is due to their impressive biosynthetic capabilities. Beside the large set of enzymes (dehydrogenases, peroxidases, oxygenases, alkylsulphatases, nitrilhydratases, and phenolhydrolases) the *Rhodococcus* spp. produce glycolipid biosurfactants and bio-flocculation agents, especially acrylamide, triacylglycerols, carotenoids, and polyhydroxyalkanoates (PHAs) [3, 7, 8]. The metabolic versatility of rhodococci makes them well-equipped for industrial uses, such as biotransformation and the biodegradation of xenobiotic compounds [8, 9].

More recently, nanoparticles (NPs) of different nature are used to enhance the biodestructive capabilities of the rhodococci [10–14]. Particular

attention is paid to the iron oxide NPs (IONs), which, besides having a large surface area to volume ratio, possess such unique properties as superparamagnetism and biocompatibility. Most commonly, magnetite NPs are used to immobilize cells of microorganisms. This method has two main advantages: it enhances the biodegradation activity of immobilized *Rhodococcus* spp. and facilitates the recovery and reuse of cells decorated with magnetic NPs [10–13].

Iron NPs have no inhibitory and adverse effect on *Rhodococcus* spp. cells growth and survival, however bacteria exhibit various metabolic responses upon interaction with iron NPs, and the potential applications of IONs depends on their physicochemical characteristics and surface properties of bacterial cells as well as environmental/culture conditions [11, 13]. It is well known that in biological media iron NPs are oxidized, generating reactive oxygen species (ROS), which are responsible for inducing oxidative stress in living organisms [15, 16]. Whatever the conditions of stress, microorganisms respond by starting the mechanisms of the phenomenon of cellular heterogeneity, meant to ensure the survival of the population [5, 17, 18]. Bacteria of the genus *Rhodococcus* are not an exception [19, 20].

Our laboratory holds the *Rhodococcus rhodochrous* CNMN-Ac-05 strain, which is a destructor of benzothiazole and its metabolites [21]. Combination of *R. rhodochrous* CNMN-Ac-05 with magnetite NPs would allow recovering and reusing of the strain in several cycles of degradation. For this reason, the purpose of our research was to determine the influence of magnetite NPs on the viability and colony morphology of *R. rhodochrous* CNMN-Ac-05 strain.

Materials and methods

Chemicals. Iron(II) sulfate (≥ 99.7 %), a saturated iron(III) chloride solution (≥ 99.0 %), poly-*N*-vinylpyrrolidone (PVP, MW: 8000), and ammonium hydroxide (≥ 99.9 %) were purchased from Sigma-Aldrich. All chemicals used in this study were of analytical grade and commercially available.

Nanomaterials. Encapsulated magnetite (Fe_3O_4) NPs were synthesized by chemical co-precipitation method, using iron(II) sulfate and iron(III) chloride in the presence of poly-*N*-vinylpyrrolidone (PVP) used as a stabilizer [22]. Fe_3O_4 SR (Synthesis in the Reactor) was produced in the multifunctional reactor VGR-50, at the same conditions.

Bacterial strain and culture conditions. *R. rhodochrous* CNMN-Ac-05 was deposited in the National Collection of Non-Pathogenic Microorganisms of the Republic of Moldova, and was able to degrade benzothiazoles, persistent organic pollutants [21]. *R. rhodochrous* CNMN-Ac-05 was grown in 100 mL portions of Tryptic soy (TS) broth (Sigma-Aldrich) in 300 mL Erlenmeyer flasks incubated at 28 °C and 200 rpm. The cells were harvested after 36 h of culture and centrifuged at 6.000 rpm for 20 min. The bacterial pellet was washed first with a NaCl solution (0.8 %) and then with distillated water.

Determination the effects of magnetite. Bacterial biomass was resuspended in distillated water (pH 7.2) to prepare cell suspension 6 mg/mL (1.4 mg of cell dry weight/mL). Colloidal aqueous solutions of magnetite, 2 mg/mL, were prepared on ultrasonic cleaner at 50 kHz for 5 min. For experiments, 5 mL of bacterial cells suspension was added in 250 mL Erlenmeyer flasks containing 95 mL of PAS medium and Fe_3O_4 or Fe_3O_4 SR in the following concentrations (mg/L): 1, 10, 25, 50, and 100. The PAS medium contained (g/L): K_2HPO_4 – 4.35, KH_2PO_4 –1.7, NH_4Cl – 2.1, $MgSO_4$ – 0.2, $MnSO_4$ – 0.05, $FeSO_4 \cdot 7H_2O$ – 0.01, $CaCl_2 \cdot 2H_2O$ – 0.03 [23]. pH adjusted at 7.2. Inoculated flasks were incubated in a rotary shaker (180 rpm) at 28 °C for 24 h. After the serial dilution the 50 µL of suspension was spread on agar plates with TS medium, the plates were incubated at 28 °C for 96 h, for the appearance of bacterial colonies. The number of viable bacterial cells was estimated by colony-forming units (CFU) inoculated on agar plates.

Cell biomass was determined on the spectrophotometer by the optical density at 540 nm, with subsequent recalculation to cell dry weight according to the calibration curve. The cell dry weight was determined by gravimetric method, drying biomass at 105 °C.

The morphological features of the rhodococci colonies were described according to method [24] using a magnifying glass (8-fold magnification).

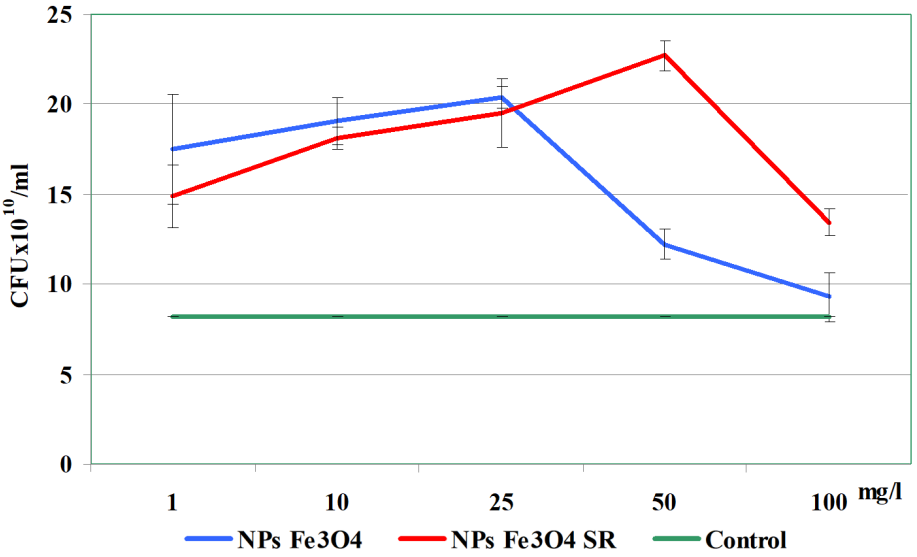
Statistical analysis was performed using MS Excel. All results were expressed as mean of three individual replicates ± CI (confidence intervals). All differences were considered significant at $P<0.05$.

Results. Testing of the action of nanomagnetite, obtained by different ways (laboratory method

and in the reactor), on the viability of the *R. rhodochrous* CNMN-Ac-05 strain showed that regardless of the method used, magnetite NPs stimulates the growth of rhodococci cells. However, there was a difference in the response of rhodococci to the presence of magnetite NPs.

Thus, the optimal concentration of Fe_3O_4 NPs, which stimulated the growth and development of the *R. rhodochrous* strain with 147.8 % compared to the control, was 25 mg/L (Fig. 1). At higher concentrations of Fe_3O_4 NPs the viability of bacterial cells decreased considerably by 1.7 times (50 mg/L) and 2.2 times (100 mg/L), but exceeded the control by 50 % and 13 % respectively.

At concentrations of 1–25 mg/L the Fe_3O_4 SR NPs also stimulated the growth of *Rhodococcus* cells, but CFU values were on 11–32 % lower, compared with Fe_3O_4 NPs. The most favorable concentration of Fe_3O_4 SR NPs for the growth and multiplication of rhodococci was 50 mg/L, the cell biomass exceeded the control by 176.1 %. But doubling this concentration led to a significant decrease in the number of bacterial cells, however the CFU values exceeded the control by 63 % (Fig. 1)



F i g. 1. Influence of magnetite NPs on the viability of *R. rhodochrous* CNMN-Ac-05 strain

In addition to cells multiplication, the morphological modification of *R. rhodochrous* CNMN-Ac-05 colonies under magnetite NPs was established. Macroscopically observable features and colony types of *Rhodococcus* are presented in Table 1 and Fig. 2.

Basic morphological features of *R. rhodochrous* CNMN-Ac-05 colonies corresponded to type S1 and this type was predominant in all experimental variants. Regardless of the nanomaterial used, the share of S1 type colonies varies in the range of 99.1–100 %. On TS medium without NPs (control variant) type S1 made up 100 %.

In the presence of Fe_3O_4 NPs, in all experimental variants appeared colonies of morphotype R1, which made up 0.3–0.5 % of the population (Table 2). The addition of Fe_3O_4 NPs in concentration of 100 mg/L led not only to the decrease in the number of CFU, but also to the dissociation of the strain in 4 types of colonies S1, S2, R1 and S-R, however, the basic type S1, remained predominantly – 99.5 %.

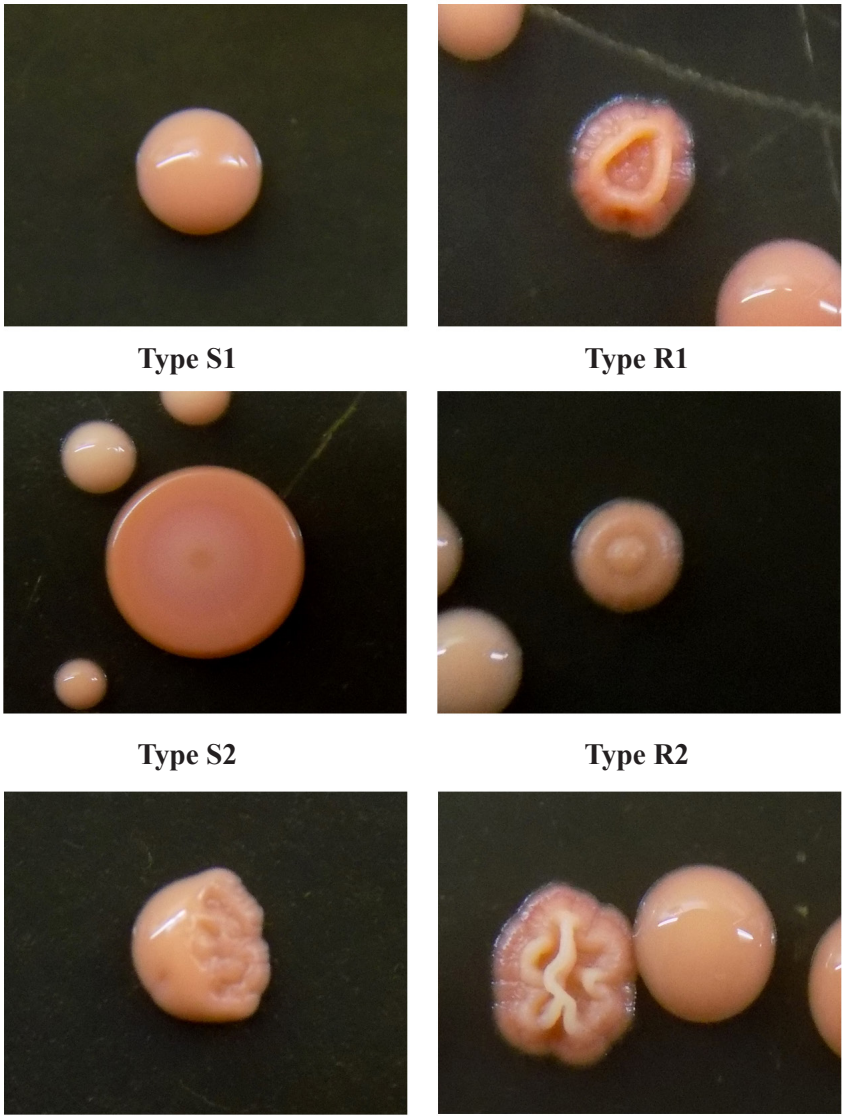
Rhodococci growth in the presence of Fe_3O_4 SR NPs also led to the appearance of new types of colonies, but not in all experimental variants (Table 2). At concentrations of 25 and 50 mg/L the bacterial population remained homogeneous, being composed of 100 % S1 type colonies. At the concentration of 10 mg/mL, the appearance of R1 and S-R types was registered; and increase in concentration to 100 mg/L of Fe_3O_4 SR NPs led not only to the sudden decrease in the number of CFU, but also to the dissociation of the *Rhodococcus* population in 4 types of colonies – S1, R1, R2 and S-R.

Discussion. Traditionally, ecosystems and microbial communities have been studied as an ensemble of different monoclonal populations. It is considered that each population is composed of genetically identical cells that perform the same metabolic function. The development of single-cell resolution techniques has made it possible to highlight cell fractions in an isogenic

Table 1

Colony morphotypes formed by *R. rhodochrous* CNMN-Ac-05 strain

Colony morpho type	Form	Size, mm	Margin	Elevation	Surface	Color	Opacity
S1	Round	1.0–4.0	Entire	Convex	Smooth and glistening	Pink	Opaque
S2	Round	3.0–3.5	Entire	Umbonate	Smooth and glistening	The center light pink, the edges dark pink	Opaque
R1	Irregular	1.0–4.0	Undulate	Convex	Rough and dull	Pink	Opaque
R 2	Round	2.0	Entire	Umbonate	Wrinkled and dull	Pink	Opaque
S-R	Round / Irregular	2.5–3.0	Entire/ Undulate	Convex	Smooth /Rough and Glistenin / Dull	Pink	Opaque



Type S1 **Type R1**

Type S2 **Type R2**

Type S–R **Types R1 and S1**

F i g. 2. Morphological modification of colonies formed by strain *R. rhodochrous* CNMN-Ac-05 cultivated in the presence of magnetite NPs

Table 2

The variation in colony morphology of *R. rhodochrous* CNMN-Ac-05 strain grown in the presence of Fe_3O_4 NPs

Concentration of magnetite NPs, mg/L	Types of colonies	Share, %	
		Fe_3O_4 NPs	Fe_3O_4 SR NPs
Control	S1	100	100
1.0	S1	99.6	99.5
	R1	0.4	0.5
10.0	S1	99.5	99.1
	R1	0.5	0.6
	S-R	–	0.3
25.0	S1	99.7	100
	R1	0.3	–
50.0	S1	99.6	100
	R1	0.4	–
100.0	S1	99.5	99.6
	S2	0.13	–
	R1	0.24	0.2
	R2	–	0.1
	S-R	0.13	0.1

Legend: Meaning of abbreviations see in Table 1.

population that behaves differently from others, even if environmental conditions have not changed significantly. The phenomenon, when genetically identical cells manifest cell-to-cell variability, even when sharing the same environmental and nutritional conditions, is known as phenotypic heterogeneity [25, 26].

Phenotypic heterogeneity is a very important means by which many bacterial populations adapt to changing environments [17, 26]. During its evolution, bacteria have developed multiple strategies to cope with rapid and frequent changes in their environment: spore formation, changes in polysaccharide production, motility or in metabolic capacity, antibiotic response and many more [26].

The phenomenon of phenotypic heterogeneity is quite common in coryneform and nocardioform bacteria. The splitting of a homogeneous rhodococci population into variants with different morphological, physiological, biochemical, and genotypic properties have been observed by many researchers. For example *R. opacus* 1CP strain grown on chlorophenols has dissociated on two morphologically and physiologically distinct variants and a labile transitional variant, which was an intermediate case [19, 27]. Studying of the ability of actinobacteria *R. opacus* 1CP to survive under unfavorable conditions (starvation, oxidative stress, low temperature (4 °C), and dehydration) has revealed that general strategy for survival was decreased cell size/volume and formation of densely-packed cell conglomerates [20]. Modifications in cell viability, cell morphology, membrane permeability, lipid profile, carotenoid pigments profile and 16S rRNA gene were revealed for *R. erythropolis* cells grown in the presence of 1 % alkanes (cyclohexane, *n*-hexane, *n*-decane) and aromatics (toluene, styrene, ethylbenzene) [28]. In our studies, the appearance of colonies of type R and altercolor occurred after the contact of *R. rhodochrous* with various solid matrices, which were used for microorganism's immobilization. Moreover, the frequency of dissociation and the degree of variability of bacteria depended on the nature of the substrate [29].

A relationship between colony morphotypes formed by *R. rhodochrous* and its tolerance to aromatic substances was demonstrated [30]. Since the ability to exhibit a high level of metabolic activity is linked with the nature of bacterial growth and belonging to a certain morphological type, it is important to determine the nature of the effect of NPs on the growth and heterogeneity of the studied bacterial culture.

In our research, magnetite NPs had a positive effect on the viability of *R. rhodochrous* CNMN-Ac-05 cells. In all experimental variants, regardless of the NPs used and their concentration, growth stimulation was established, and the amount of biomass exceeded the control values. However, the

maximum peak of biomass accumulation differs depending on the concentration and the method of preparing of magnetite NPs, in the case of NPs Fe_3O_4 the optimal concentration was 25 mg/L, and in the case of NPs Fe_3O_4 SR – 50 mg/L.

There are also differences in colony dissociation of the *Rhodococcus* population. In the presence of Fe_3O_4 NPs in all experimental variants, along with the basic type S1, colonies of the R1 type appeared. At the concentration of 100 mg/L, together with a considerable biomass reduction, the strain dissociated in 4 types of colonies – S1, S2, R1, and S-R.

The effect of Fe_3O_4 SR NPs on natural variability of rhodococci colony morphotypes did not correlate with the concentration. As in the previous case, the increase in concentration to 100 mg/L coincided with the significant decrease in biomass and the highest degree of dissociation of the bacterial population (4 types of colonies – S1, R1, R2 and S-R).

Conclusions. In conclusion the optimal concentration of magnetite NPs, which stimulated the growth and development of the *R. rhodochrous* was 25 mg/L for Fe_3O_4 and 50 mg/L for Fe_3O_4 SR. However, it should be noted that in the presence of magnetite NPs the variation in colony morphology of the rhodococci was quite low; the new types of colonies represented only 0.1–0.6 % of the population, and the lowest degree of variability corresponded with the highest CFU index. This allows us to conclude that the studied magnetite NPs, in concentrations of 1–100 mg/L, were not toxic to *R. rhodochrous* CNMN-Ac-05 strain, and can be used to obtain bio-nano-systems.

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ЖИТТЄЗДАТНІСТЬ І МОРФОЛОГІЧНА ВАРІАБЕЛЬНІСТЬ КОЛОНІЙ *RHODOCOCCUS RHODOCHROUS* CNMN-AC-05 У ПРИСУТНОСТІ НАНОЧАСТИНОК МАГНЕТИТУ

***О. Постолакі*[1], *І. Растімєшіна*[1], *В. Жосан*[1], *Т. Гуцул*[2]**

[1]Інститут мікробіології та біотехнології, Вул. Академічна, 1, Кишинів, MD-2028, Республіка Молдова
[2]Інститут електронної інженерії та нанотехнологій ім. Д. Гіцу, Вул. Академічна, 3/3, Кишинів, MD-2028, Республіка Молдова

Резюме

За останні десятиліття використання нанотехнологій для детоксикації ксенобіотиків довело свою ефективність, але не безпеку. Наночастинки часто накопичуються у середовищі та з часом починають токсично впливати на живі організми. У цьому контексті дослідження життєздатності мікроорганізмів за умов їх взаємодії з наночастинками має важливе значення. **Метою** досліджень було визначити вплив наночастинок Fe_3O_4, отриманих різними способами (лабораторним методом та шляхом синтезу у реакторі), на життєздатність та морфологію колоній штаму *R. rhodochrous* CNMN-Ac-05. **Методи.** Наночастинки інкапсульованого магнетиту (Fe_3O_4) синтезували хімічним методом спільного осадження з використанням сульфату заліза (II) та хлориду заліза (III) у присутності полі-N-вінілпіролідону, який використовували в якості стабілізатору. Fe_3O_4 SR (синтезовані в реакторі) отримували в багатофункціональному реакторі VGR-50 за тих самих умов. Біомасу клітин визначали на спектрофотометрі за оптичною щільністю при 540 нм з подальшим перерахунком на суху масу клітин згідно калібрувальної кривої. Суху масу клітин визначали гравіметричним методом. Морфологічні особливості колоній *Rhodococcus* описували за звичайним мікробіологічним методом. **Результати.** Встановлено, що наночастинки магнетиту в концентраціях 1–100 мг/л не токсичні для штаму *R. rhodochrous*, позитивно впливали на життєздатність родококів, стимулюючи накопичення біомаси незалежно від їх концентрації та способу синтезу. За наявності наночастинок Fe_3O_4 популяція дисоціювала, утворюючи колонії типу S1, S2, R1, R2 та S-R, тоді як основні морфологічні ознаки колоній *R. rhodochrous* у контролі відповідали типу S1. **Висновки.** Оптимальна концентрація наночастинок магнетиту, яка стимулювала ріст і розвиток *R. Rhodochrous,* становила 25 мг/л для Fe_3O_4 та 50 мг/л для Fe_3O_4 SR. При всіх концентраціях наночастинок Fe_3O_4 основним морфотипом колоній родококів був гладкий тип S1; нові типи колоній представляли лише 0,1–0,6 % популяції, а найнижчий ступінь мінливості відповідав найвищому індексу колонієутворюючих одиниць.

Ключові слова: родококи, наночастинки магнетиту, морфологія колоній.

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